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Identification of multispecific organic anion transporter 2 expressed predominantly in the liver

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Abstract In the present study, we demonstrate that NLT (novel liver-specific transport protein) is a multispecific organic anion transporter of the liver. The amino acid sequence of NLT shows 42% identity to that of the renal multispecific organic anion transporter, OAT1. When expressed in *Xenopus laevis* oocytes, NLT mediated uptake of organic anions, such as salicylate, acetylsalicylate, PGE_2, dicarboxylates and *p*-aminohippurate. [^{14}C]Salicylate uptake via NLT was saturable ($K_m = 88.8 \pm 23.4$ μM) and sodium-independent. Expression of the mRNA of NLT was detected in the liver and kidney (liver ≫ kidney). We propose that NLT be renamed OAT2.

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Key words: Liver; Multispecific transporter; Organic anion; Pharmacokinetics

1. Introduction

The liver and kidney cells take up a number of organic anions from the plasma across their basolateral membranes [1–7]. Both similarities and differences exist between the organic anion transport systems in these two organs. Several organic anions are transported both by the liver and by the kidney, such as sulfonamides, β-lactam antibiotics, phenol red derivatives, steroid hormones and sulfonic acid dyes [8]; however, the substrate selectivity of the two systems is not identical. For example, phenolsulfophthalein and sulfobromophthalein (BSP) are excreted qualitatively into both the urine and the bile, although the former dye is predominantly excreted in the urine and the latter in bile.

So far two bile acid transporters located on the sinusoidal membrane of the hepatocytes have been identified: oatp1 (organic anion-transporting polypeptide 1) [9] and Ntcp (Na^+-taurocholate co-transporter) [10]. Interestingly, oatp1 transports several organic anions with different chemical structures, such as bile acids, BSP, conjugated steroid hormones and ochratoxin A [9,11–13], and has therefore been regarded as a multispecific organic anion transporter. However, oatp1 cannot totally account for the diverse organic anion transport in the liver [14–17].

Recently we isolated a renal organic anion transporter (OAT1) using the expression cloning method, which transports a variety of organic anions, including *p*-aminohippurate (PAH), various dicarboxylates, cyclic nucleotides, prostanoids, urate, β-lactam antibiotics, non-steroidal anti-inflammatory drugs and diuretics [18]. Independently, cDNAs encoding renal organic anion transporter of winter flounder (fROAT) [19] and rat (ROAT1) [20] were isolated. The amino acid sequence of ROAT1 is 100% identical to that of OAT1, whereas fROAT shows 46.9% identity to OAT1. OAT1 is the multispecific organic anion transporter of the basolateral membrane of the proximal tubule, whose transport properties have been extensively clarified by numerous physiological and pharmacological studies [5–7]. OAT1 mediates the elimination of endogenous and exogenous organic anions from the kidney.

A search of the DNA database revealed that the amino acid sequence of a liver-specific membrane protein, NLT (novel liver-specific transport protein), exhibits 42% identity to that of OAT1. NLT was isolated from a rat liver cDNA library using a monoclonal antibody against a glucagon receptor and its expression was limited to the sinusoidal membrane of the hepatocyte [21]. NLT possesses 12 putative transmembrane domains with specific transporter motifs and it shows a low sequence identity to sugar transporters (e.g. GLUT); however, the substrate(s) of NLT has(ve) not been identified yet [21]. Based on the sequence homology with OAT1 and its characteristic tissue distribution, we hypothesized that NLT might be a multispecific organic anion transporter of the liver.

In the present study, we demonstrated that NLT mediates sodium-independent, multispecific organic anion transport and is exclusively expressed in the liver. NLT is the second member of the multispecific organic anion transporter family.

2. Materials and methods

2.1. Isolation of NLT

A non-directional cDNA library was constructed from 3 μg of rat liver poly(A)$^+$ RNA using the Superscript Choice system (Life Technology), and was ligated to a phage vector λZipLox *Eco*RI arms (Life Technology). A PCR product corresponding to nucleotides 131–673 of NLT was labeled with ^{32}P-dCTP by random priming (T7 Quick-Prime Kit, Pharmacia) and used for the screening of a rat liver cDNA library. Replicated filters of a phage library were hybridized overnight at 37°C in a hybridization solution (50% formamide/5×standard saline citrate (SSC)/3×Denhardt's solution/0.2% SDS/10% dextran sulfate/0.3 mg/ml denatured salmon sperm DNA/2.5 mM sodium pyrophosphate/25 mM MES/0.03% Antifoam A, pH 6.5). The filters were washed finally at 37°C in 0.1×SSC/0.1% SDS. Positive cDNA inserts in λZipLox phage vector were recovered into the plasmid pZL1 by in vitro excision.

2.2. Sequence of NLT

Specially synthesized oligonucleotide primers were used for sequencing of the cDNA obtained by the screening. Sequencing was performed by the dideoxy termination method.

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2.3. cRNA synthesis and uptake experiment using Xenopus laevis oocytes

The plasmid DNAs in the pZL were used for in vitro transcription. Capped cRNAs were synthesized in vitro using T7 RNA polymerase from the linearized plasmid DNAs with *Bam*HI. Defolliculated oocytes were injected with 10 ng of capped cRNAs and incubated in Barth's solution containing gentamicin (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, gentamicin 50 μg/ml, pH 7.4) at 18°C. After incubation for 2–3 days, uptake experiments were performed in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) containing radiolabeled substrates, as indicated in each experiment, at room temperature.

2.4. Northern blot analysis

Three microgram of poly(A)$^+$ RNA prepared from various rat tissues was electrophoresed on a 1% agarose/formaldehyde gel and transferred onto a nitrocellulose filter. The filter was hybridized in a hybridization solution overnight at 42°C with a full-length cDNA of NLT which was randomly labeled with ^{32}P-dCTP. The filter was washed finally in 0.1×SSC/0.1% SDS at 65°C.

3. Results

3.1. Isolation and sequencing of NLT

Using the ^{32}P-labeled NLT fragment as a probe, we isolated six positive cDNA clones from the rat liver cDNA library. Three clones were presumed to contain the total coding region of NLT, from which we synthesized the cRNAs in vitro. When expressed in *Xenopus laevis* oocytes, two of these clones (clones L1 and L5) mediated the uptake of [^{14}C]α-glutarate. In the following experiments we further analyzed clone L5. The nucleotide sequence of L5 is identical to that of NLT except one base change (G at position 1509 to C) resulting in the change of an amino acid (Arg^{479} to Pro). L5 lacks nucleotides 1–23 of NLT, and contains 13 poly(A)$^+$.

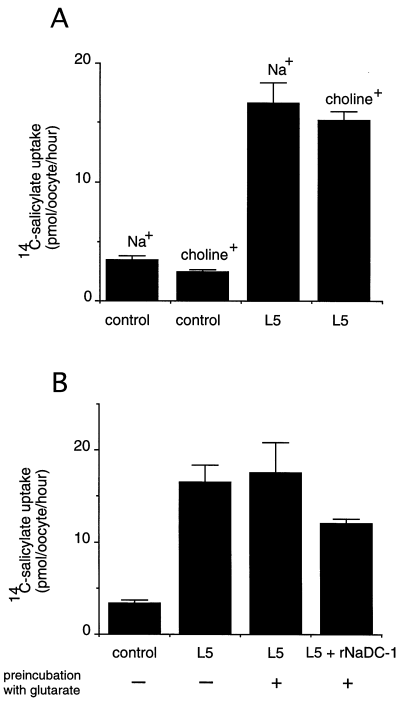

Fig. 1. [^{14}C]Salicylate uptake in *Xenopus laevis* oocytes expressing L5. A: Independence of L5-mediated [^{14}C]salicylate (10 μM) uptake of extracellular Na$^+$. B: Independence of L5-mediated [^{14}C]salicylate uptake of intracellular glutarate concentration. Oocytes are expressed with L5 or L5 and rNaDC-1. 10 μM [^{14}C]salicylate uptake was determined with or without the preincubation of the oocytes with 1 mM glutarate.

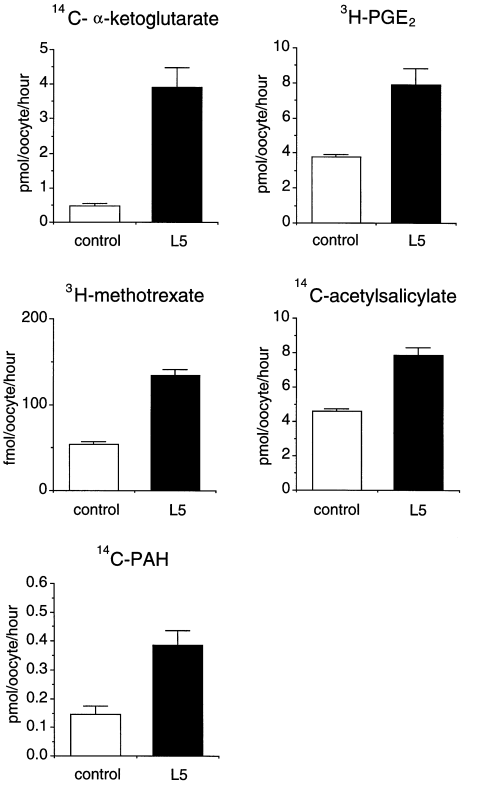

Fig. 2. L5-mediated uptake of radiolabeled organic anions. The uptake rates of radiolabeled compounds ([^{14}C]α-ketoglutarate: 7 μM, [^{3}H]prostaglandin E_2: 6 nM, [^{3}H]methotrexate: 1 μM, [^{14}C]acetylsalicylate: 20 μM, [^{14}C]PAH: 20 μM) by control (open column) or L5-expressed (closed column) oocytes were measured for 1 h (mean ± S.E.M.; n = 5–8).

3.2. Functional characterization

Oocytes injected with L5 cRNA mediated the transport of [^{14}C]salicylate in a sodium-independent manner (Fig. 1A). Since the renal multispecific organic anion transporter, OAT1, is an organic anion/dicarboxylate exchanger [18], we examined whether dicarboxylic acid *trans*-stimulates the organic anion uptake via L5. As previously described [18], we co-expressed L5 and rat Na$^+$-dicarboxylate transporter (rNaDC-1) in oocytes and performed the uptake experiment. The uptake rate of [^{14}C]salicylate via L5 was measured after preincubating the oocytes with 1 mM glutarate, which induces an outwardly directed dicarboxylate gradient. If L5 was an organic anion/dicarboxylate exchanger, intracellularly accumulated glutarate, via rNaDC-1, would stimulate the uptake of [^{14}C]salicylate via L5. As shown in Fig. 1B, the outwardly directed gradient of glutarate did not enhance the uptake rate of [^{14}C]salicylate via L5.

As shown in Fig. 2, L5 mediated the transport of several organic anions with different chemical structures, such as [^{14}C]α-ketoglutarate, [^{3}H]prostaglandin E_2, [^{3}H]methotrexate and [^{14}C]acetylsalicylate. L5 also mediated a low level transport of [^{14}C]PAH, which is a prototype substrate for the renal organic anion transport system. In contrast, L5 did not transport [^{14}C]tetraethylammonium, which is a typical substrate for the organic cation transport system of the kidney (data not

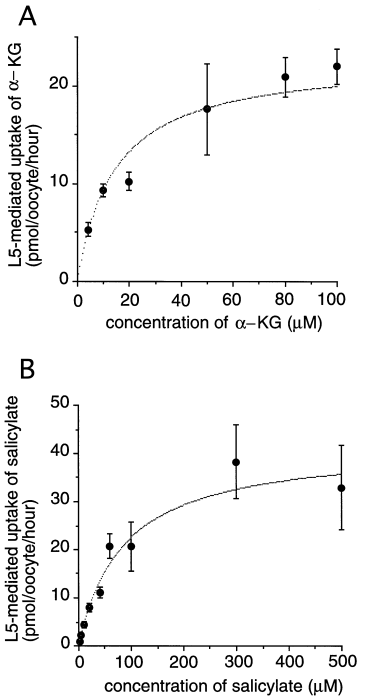

Fig. 3. Concentration dependence of L5-mediated uptake of [^{14}C]α-ketoglutarate and [^{14}C]salicylate.

shown). L5 did not mediate the transport of either cholate, taurocholate or estrone sulfate (data not shown).

We analyzed the transport kinetics of α-ketoglutarate and salicylate via L5. Fig. 3 shows the concentration-dependent uptake of [^{14}C]α-ketoglutarate and [^{14}C]salicylate via L5. Transport of α-ketoglutarate and salicylate via L5 exhibits saturable kinetics and follows the Michaelis-Menten equation. The calculated K_m values of α-ketoglutarate and salicylate were 17.8 ± 2.9 μM and 88.8 ± 23.4 μM, respectively.

In order to further evaluate the substrate specificity of L5, we performed an inhibition study in oocytes expressing L5. The uptake of 1 μM [^{14}C]salicylate via L5 was determined in the presence or absence of 1 mM test substrates; the concentration of BSP used was 250 μM because of its cytotoxic effect. As shown in Fig. 4, BSP, indocyanin green (ICG), cholic acid, bumetanide, cefoperazone, rifampicin and ketoprofen exerted inhibitory effects on the transport of salicylate via L5.

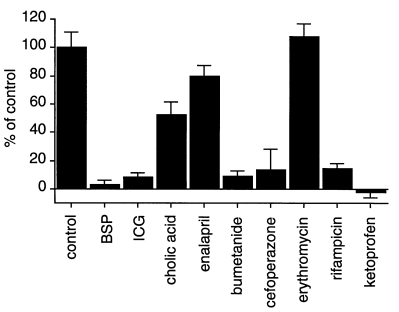

Fig. 4. Inhibition of L5-mediated [^{14}C]salicylate uptake by various compounds. The concentration of [^{14}C]salicylate was 1 μM and those of inhibitors were 1 mM except BSP (250 μM). The values are expressed as a percentage of L5-mediated [^{14}C]salicylate uptake without the inhibitor (mean ± S.E.M.; n = 5–8 oocytes).

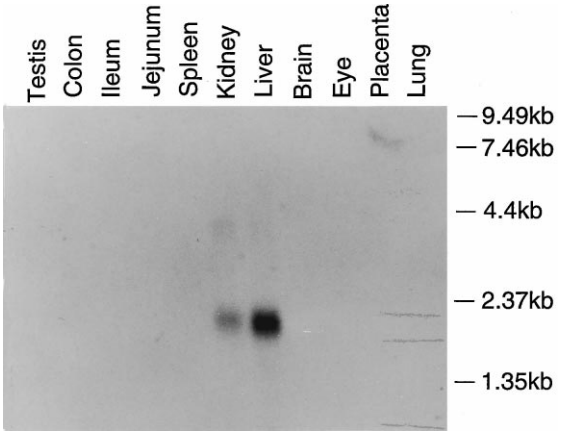

Fig. 5. Northern blot analysis of L5 in rat tissues. 3 μg of poly(A)$^+$ RNAs from various rat tissues were probed with ^{32}P-labeled cDNA of L5.

3.3. Northern blot analysis

We investigated the tissue distribution of L5 (Fig. 5). High-stringency Northern blot analysis revealed that mRNA expression of L5 is liver-specific. A transcript (2.0 kb) was detected strongly in the liver and weakly in the kidney. No positive signal for the mRNA was detected in other rat tissues, including the testis, small and large intestines, spleen, brain, eye, placenta or lung.

4. Discussion

Organic anions represent a group of chemically heterogeneous compounds which possess a carbon backbone and carry a net negative charge at physiological pH, and include a large number of endogenous (e.g. bile acids, bilirubin and fatty acids) and exogenous substances (anionic drugs, carcinogens, food additives and environmental toxins). Hepatocytes take up these organic anions from the plasma and secrete them into bile [1–4]. Organic anions, besides being transported, are also actively produced within the liver. Lipophilic compounds which enter hepatocytes by passive diffusion are subjected to phase I and phase II reactions, resulting in the production of various organic anions, which are not only secreted into bile but also refluxed into the blood stream. Thus, the sinusoidal membranes of hepatocytes are continuously involved in the bidirectional transport of organic anions [4]. Recent physiological studies have indicated that the transport of a large number of organic anions across the sinusoidal membrane of the hepatocytes is mediated by multispecific organic anion transporter(s) [1–3].

Bile acids are the major organic anions secreted from the liver, and undergo enterohepatic circulation [2]. Owing to its physiological importance, the transport of bile acids in the liver has been extensively investigated. Recently, two bile acid transporters have been isolated using the expression cloning method: Ntcp [10] and oatp1 [9]. In contrast to Ntcp which transports only bile acids, oatp1 has been shown to be a multispecific transporter. Oatp transports not only bile acids, but also conjugated steroid hormones, several anionic dyes (such as BSP) and ochratoxin A [9,11–13]. The diversity of hepatocellular uptake of organic anions across the sinusoidal membrane was therefore attributed to oatp1. However, it soon became apparent that oatp1 does not mediate the

transport of characteristic substrates of the organic anion transport system of the sinusoidal membrane of the liver [16,17]. Oatp1-mediated uptake of BSP was not *cis*-inhibited by organic anions such as bumetanide, dicarboxylic acids, rifampicin, LTC4 and reduced GSH [14]. So far, three isoforms of oatp1, that is oatp2 [22], OAT-K1 [23] and prostaglandin transporter (PGT) [24], have been isolated. Oatp2 exhibits similar substrate selectivity as oatp1. OAT-K1 and PGT transport methotrexate and prostaglandins, respectively. The isoforms of oatp also do not explain the transport of a variety of organic anions in the liver.

From the observation of the structural similarity of NLT to the renal multispecific organic anion transporter OAT1, we investigated the organic anion transport via NLT and demonstrated that NLT mediates multispecific organic anion transport. The substrate selectivity of NLT and OAT1 appears similar, however, the transport efficacy of each substrate is different. Previous physiological experiments have revealed that PAH is excreted mainly into the urine and scarcely into the bile. On the other hand, salicylate is taken up by both the liver and the kidney. The transport of PAH by NLT occurs at a much lower level than that by OAT1 [18]; in contrast, the transport of salicylate by NLT occurs at a higher level than that by OAT1 (unpublished data). The different transport efficacy of NLT and OAT1 may reflect the different tissue distribution of organic anions in the body. For examples, β-lactam antibiotics are usually excreted into the urine by renal proximal tubule cells via the organic anion transport pathway; however, several drugs exist which are excreted mainly into the bile, e.g. cefoperazone and piperacillin. Nearly all of the β-lactam antibiotics are very hydrophilic compounds and their efficient uptake into the liver requires the mediation of carrier(s). The difference in pharmacokinetics among the β-lactam antibiotics may be explained by the different substrate selectivity and the specific tissue distribution of OAT1 and NLT. Bumetanide is a diuretic which is taken up by both the kidney and the liver. It has been reported that bumetanide is transported not by oatp [17,25], but by the kidney-like organic anion transport system in the liver. NLT may also be responsible for the hepatocellular uptake of bumetanide. Further functional analyses are required for the clarification of the role of NLT in organic anion transport in the liver.

The transport of organic anions has been demonstrated in other organs, such as the ciliary body, lung, placenta, choroid plexus and intestine [5]. Recently the transport of PAH through the blood-brain barrier was demonstrated [26]. When PAH was microinjected into a cerebral cortex region termed Par2, it was extensively eliminated from the ipsilateral cerebrum and this transport of PAH showed a saturation kinetic with a Michaelis constant of 400 μM. Nonetheless, the studies of the organic anion transport in these tissues are limited because of technical difficulties. We hypothesized the existence of other members of the multispecific organic anion transporter family (OAT family) in these tissues and have already identified several. Identification of all the members may help to clarify why certain organic anions are preferentially distributed in certain organ(s).

Salicylate uptake via NLT is sodium-independent like that via OAT1. In the liver as well as the kidney, the presence of a dicarboxylate/organic anion exchange system has been predicted [27]. In the present study however, we could not demonstrate the *trans*-stimulatory effect of glutarate on the uptake of salicylate. Although only from this evidence it is difficult to conclude that NLT is not an exchanger, NLT may function as a facilitatory transporter rather than an exchanger. Thus the transport properties of NLT remain to be elucidated.

In conclusion, we demonstrated that the liver-specific transporter NLT mediates the transport of structurally unrelated organic anions. The amino acid sequence of NLT exhibits 42% identity to that of a renal multispecific organic anion transporter OAT1. NLT is the second member of the OAT family and we propose that NLT be renamed OAT2 (organic anion transporter 2).

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References

[1] Meier, P.J. (1995) Am. J. Physiol. 269, G801–812.
[2] Petzinger, E. (1994) Rev. Physiol. Biochem. Pharmacol. 123, 47–211.
[3] Boyer, J.L., Graf, J. and Meier, P.J. (1992) Annu. Rev. Physiol. 54, 415–438.
[4] Tiribelli, C., Lunazzi, G.C. and Sottocasa, G.L. (1990) Biochim. Biophys. Acta 1031, 261–275.
[5] Pritchard, J.B. and Miller, D.S. (1993) Physiol. Rev. 73, 765–796.
[6] Moller, J.V. and Sheikh, M.I. (1983) Pharmacol. Rev. 34, 315–358.
[7] Ullrich, K.J. and Rumrich, G. (1993) Clin. Invest. 71, 843–848.
[8] Despopoulos, A. (1971) Am. J. Physiol. 220, 1755–1758.
[9] Jacquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A.W. and Meier, P.J. (1994) Proc. Natl. Acad. Sci. USA 91, 133–137.
[10] Hagenbuch, B., Stieger, B., Foguet, M., Lubbert, H. and Meier, P.J. (1991) Proc. Natl. Acad. Sci. USA 88, 10629–10633.
[11] Kanai, N., Lu, R., Bao, Y., Wolkoff, A.W., Vore, M. and Schuster, V.L. (1996) Am. J. Physiol. 270, F326–331.
[12] Bossuyt, X., Muller, M., Hagenbuch, B. and Meier, P.J. (1996) J. Pharmacol. Exp. Ther. 276, 891–896.
[13] Kontaxi, M., Echkardt, U., Hagenbuch, B., Stieger, B., Meier, P.J. and Petzinger, E. (1996) J. Pharmacol. Exp. Ther. 279, 1507–1513.
[14] Kullak, U.G., Hagenbuch, B., Stieger, B., Wolkoff, A.W. and Meier, P.J. (1994) Hepatology 20, 411–416.
[15] Kanai, N., Lu, R., Bao, Y., Wolkoff, A.W. and Schuster, V.L. (1996) Am. J. Physiol. 270, F319–325.
[16] Weinman, S.A. (1994) Hepatology 20, 1642–1644.
[17] Horz, J.A., Honscha, W. and Petzinger, E. (1996) Biochim. Biophys. Acta 1300, 114–118.
[18] Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y. and Endou, H. (1997) J. Biol. Chem. 272, 18526–18529.
[19] Wolff, N.A., Werner, A., Burkhardt, S. and Burkhardt, G. (1997) FEBS Lett. 417, 287–291.
[20] Sweet, D.H., Wolff, N.A. and Pritchard, J.B. (1997) J. Biol. Chem. 272, 30088–30095.
[21] Simonson, G.D., Vincent, A.C., Roberg, K.J., Huang, Y. and Iwanij, V. (1994) J. Cell. Sci. 107, 1065–1072.
[22] Noe, B., Hagenbuch, B., Stieger, B. and Meier, P.J. (1997) Proc. Natl. Acad. Sci. USA 94, 10346–10350.
[23] Saito, H., Masuda, S. and Inui, K. (1996) J. Biol. Chem. 271, 20719–20725.
[24] Kanai, N., Lu, R., Satriano, J.A., Bao, Y., Wolkoff, A.W. and Schuster, V.L. (1995) Science 268, 866–869.
[25] Petzinger, E., Blumrich, M., Bruhl, B., Eckhardt, U., Follmann, W., Honscha, W., Horz, J.A., Muller, N., Nickau, L., Ottallah, K.M., Platte, H.D., Schenk, A., Schuh, K., Schulz, K. and Schulz, S. (1996) J. Hepatol. 24, (Suppl. 1) 42–46.
[26] Kakee, A., Terasaki, T. and Sugiyama, Y. (1997) J. Pharmacol. Exp. Ther. 283, 1018–1025.
[27] Boelsterli, U.A., Zimmerli, B. and Meier, P.J. (1995) Am. J. Physiol. 268, G797–805.